Cell

Neuronal Subtype Specification within a Lineage by Opposing Temporal Feed-Forward Loops

Magnus Baumgardt,[1] Daniel Karlsson,[1] Javier Terriente,[2,3] Fernando J. Díaz-Benjumea,[2] and Stefan Thor[1,*]
[1]Department of Clinical and Experimental Medicine, Linkoping University, Linkoping SE-581 85, Sweden
[2]Centro de Biología Molecular-Severo Ochoa/C.S.I.C., Universidad Autónoma-Cantoblanco, Madrid 28049, Spain
[3]Present address: Division of Developmental Neuroscience, MRC National Institute for Medical Research, Mill Hill, London NW7 1AA, UK
*Correspondence: stefan.thor@liu.se
DOI 10.1016/j.cell.2009.10.032

SUMMARY

Neural progenitors generate distinct cell types at different stages, but the mechanisms controlling these temporal transitions are poorly understood. In the *Drosophila* CNS, a cascade of transcription factors, the "temporal gene cascade," has been identified that acts to alter progenitor competence over time. However, many CNS lineages display broad temporal windows, and it is unclear how broad windows progress into subwindows that generate unique cell types. We have addressed this issue in an identifiable *Drosophila* CNS lineage and find that a broad *castor* temporal window is subdivided by two different feed-forward loops, both of which are triggered by *castor* itself. The first loop acts to specify a unique cell fate, whereas the second loop suppresses the first loop, thereby allowing for the generation of alternate cell fates. This mechanism of temporal and "subtemporal" genes acting in opposing feed-forward loops may be used by many stem cell lineages to generate diversity.

INTRODUCTION

It is becomingly increasingly clear that progenitor cells, in both the invertebrate and vertebrate central nervous systems (CNS), undergo critical temporal transitions resulting in changes in their competence (reviewed in Jacob et al., 2008 and Okano and Temple, 2009). This is evident by the stereotyped appearance of different cell types from the same progenitor at different developmental stages. Understanding such transitions in progenitor cells is of fundamental importance for understanding cell-fate specification. However, the mechanisms controlling these temporal changes are still poorly understood.

In the *Drosophila* embryonic CNS, a serial cascade of transcription factors has been identified and found to act in most, if not all, neuroblasts to change progenitor competence over time (Figure 1I) (Brody and Odenwald, 2000; Isshiki et al., 2001; Kambadur et al., 1998; Novotny et al., 2002). Investigations of this so-called "temporal gene cascade" of *hunchback-Kruppel-pdm-castor-grainyhead* have shown that mutating or misexpressing these temporal genes result in changes of the cell types generated by that neuroblast (Cleary and Doe, 2006; Grosskortenhaus et al., 2005, 2006; Isshiki et al., 2001; Kambadur et al., 1998; Mettler et al., 2006; Novotny et al., 2002; Pearson and Doe, 2003; Tran and Doe, 2008; Tsuji et al., 2008). However, several key questions pertaining to the function of the temporal genes still remain unresolved. First, what are the downstream targets of the temporal genes? Second, how many regulatory levels away from terminal identity genes are the temporal genes (Figure 1I)? Third, many lineages are large and express temporal genes in broad windows; how are these broad windows subdivided into smaller windows that result in the generation of unique cell types (Figure 1H)? This is particularly relevant for the function of the gene *castor* (*cas*) because many large lineages appear to end with large *cas* temporal windows (Brody and Odenwald, 2000; Kambadur et al., 1998) (see below). Addressing these fundamental issues requires a number of elements: single neuroblast lineage resolution, highly selective cell type–specific markers for specific cell types within that lineage, insight into the genetic cascades acting to specify such unique cell types, and genetic tools with which to address gene function at single-lineage and single-cell resolutions.

In the embryonic *Drosophila* ventral nerve cord (VNC), the six thoracic hemisegments contain a lateral cluster of four neurons, the Ap cluster, defined by expression of the LIM-homedomain transcription factor Apterous (Ap), and the transcription cofactor Eyes absent (Eya; Figure 1A) (Lundgren et al., 1995; Miguel-Aliaga et al., 2004). Two cells in each cluster are neuropeptide-producing cells—the Ap1/Nplp1 and Ap4/FMRFa neurons, which express the neuropeptide genes *FMRFa* and *Nplp1*, respectively (Baumgardt et al., 2007; Benveniste et al., 1998; Park et al., 2004). Each Ap cluster thus contains three different cell types: the Ap1/Nplp1 neuron, two "generic" Ap cluster neurons, herein denoted Ap2 and Ap3, and the Ap4/FMRFa neuron (Figures 1A and 1B). Each developing thoracic hemisegment contains a reproducible set of 30 CNS progenitor cells, the neuroblasts, generated in seven rows (Thomas et al., 1984), and we previously determined that the Ap cluster is generated at a late stage by the lateral-most, thoracic, row five neuroblast, NB 5-6T (Figures 1F and 1G) (Baumgardt et al., 2007).

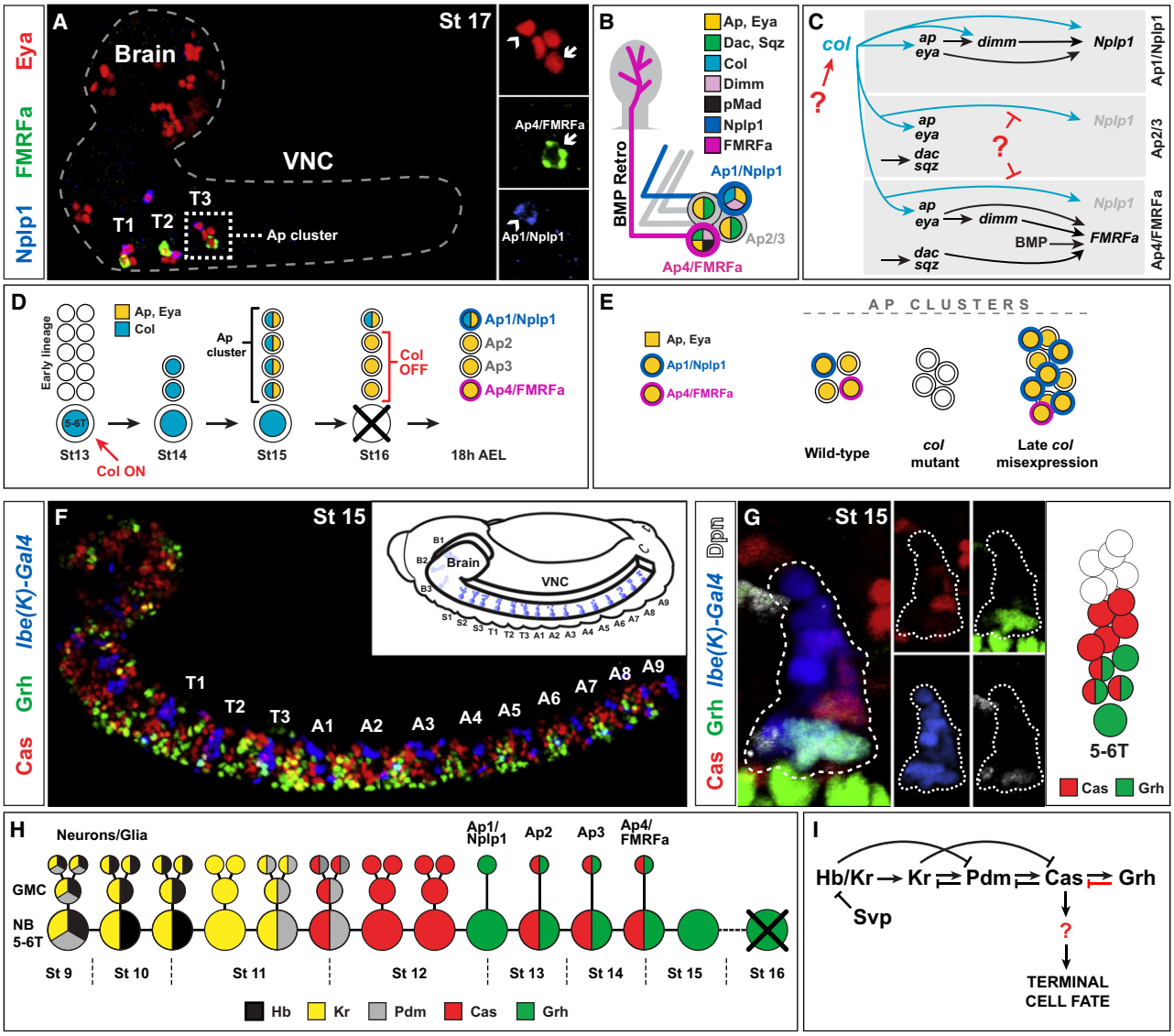


Figure 1. Specification of the Ap Cluster Neurons, and Their Lineage Origin, Thoracic Neuroblast 5-6

(A) Late embryonic *Drosophila* CNS, stained for Eya, Nplp1, and FMRFa. Expression of Eya reveals Ap clusters in the thoracic segments, with the Ap1/Nplp1 and Ap4/FMRFa neuropeptide neurons.

(B) Previous studies identified several regulatory genes specifically expressed in subsets of Ap neurons, acting to specify their identities (see text for references).

(C) Part of the genetic cascade acting to specify different Ap neurons. An unknown cue (top red arrow) triggers *col* expression late in the lineage. *col* plays a critical early role in establishing a "generic" Ap neuron fate in all four Ap neurons, by activating *ap* and *eya*. *col* subsequently acts in a feed-forward loop to specify the Ap1/Nplp1 cell fate. An unknown cue (central red arrows) acts to down-regulate *col* in the three later-born Ap neurons—Ap2/3 and Ap4/FMRFa. *dac* is activated by an unknown mechanism.

(D) The dynamics of Col expression within the NB 5-6T lineage, showing the two critical steps in Col regulation; Col ON and Col OFF.

(E) In *col* mutants, Ap neurons are generated but not properly specified. Late *col* misexpression leads to misspecification of earlier-born cells in the NB 5-6T lineage into Ap neurons, specifically into Ap1/Nplp1 and Ap2/3 fate.

(F) Expression of *lbe(K)-Gal4* reveals the NB 5-6 lineage in all CNS segments. Expression of Cas and Grh is evident in intermediate and ventral-most layers of the CNS.

(G) Lateral view of the NB 5-6T lineage, showing expression of Cas, Grh, and Dpn. Dorsal-most cells do not express the late temporal genes Cas and Grh, which are expressed in intermediate and ventral-most parts of the lineage, respectively. Dpn marks the neuroblast and the last-born GMC/neuron.

(H) Model of the NB 5-6T lineage. NB 5-6T undergoes eight typical asymmetric divisions, generating secondary progenitor cells (GMCs) that divide once to generate neurons or glia. At stage 13, there is a switch in the mode of division, and the neuroblast "buds off" four consecutive neurons, without GMC intermediates, before exiting the cell cycle. At stage 16, the neuroblast undergoes apoptosis. The four Ap cluster neurons are the last-born neurons, and are born within a large Cas window, that also expresses Grh.

(I) Model of the temporal gene cascade and regulatory relationship between the temporal genes.

Genotype: w^{1118} in (A) and *lbe(K)-Gal4* and *UAS-nmEGFP* in (F) and (G).

Several genes have been identified that specify Ap cluster neurons and that regulate Nplp1 and FMRFa. These include genes encoding Ap itself, the COE class transcription factor Collier/Knot (Col), the zinc-finger protein Squeeze (Sqz), the bHLH protein Dimmed (Dimm), the zinc-finger homeodomain protein Zfh1, as well as the Dachshund (Dac), Chip, Nab, and Eya transcription cofactors (Allan et al., 2003, 2005; Baumgardt et al., 2007; Benveniste et al., 1998; Hewes et al., 2003; Miguel-Aliaga et al., 2004; Park et al., 2004; Terriente Felix et al., 2007; van Meyel et al., 2000; Vogler and Urban, 2008). In addition, expression of FMRFa depends on a target-derived TGF-β/BMP retrograde signal (Figure 1B) (Allan et al., 2003; Marques et al., 2003). Genetic analysis reveals that these genes act in three different regulatory cascades to dictate Ap1/Nplp1, Ap2/3, and Ap4/FMRFa cell identity (Figure 1C). Col plays a central role during Ap neuron specification and is expressed by all four early-born Ap neurons, where it acts to activate *ap* and *eya*. Col then acts in a *col*→*ap/eya*→*dimm*→*Nplp1* feed-forward loop in the Ap1/Nplp1 cell to specify this cell fate (Baumgardt et al., 2007). However, Col is rapidly down-regulated in the Ap2/3 and Ap4/FMRFa neurons, and this down-regulation is critical to allow these later-born neurons to adopt their distinct cell fates (Figures 1C–1E). *sqz* plays a complex role in Ap neuron specification and was referred to as controlling "Ap cluster composition" (Allan et al., 2005). Sqz interacts physically with the well-conserved transcriptional cofactor Nab (Terriente Felix et al., 2007), but the expression and precise role of *nab* during Ap cluster specification has not been resolved. *dac*, in turn, is important for specifying the Ap4/FMRFa cell fate (Miguel-Aliaga et al., 2004). From these previous studies, three key questions emerged. First, within the NB 5-6T lineage, expression of Col, Dac, and *sqz* is triggered specifically in the Ap window—what is the upstream temporal cue? Second, how is the down-regulation of *Col* from Ap2/3 and Ap4/FMRFa controlled? Finally, the Col feed-forward loop specifies Ap1/Nplp1 fate, and the absence of Col specifies the Ap2/3 fate, but how is the Ap4/FMRFa fate specified (Figure 1C)?

Here, we find that the Ap cluster neurons are the last four cells to be born, in the comparatively large NB 5-6T lineage of 20 cells. Ap cluster neurons are not sibling cells and, surprisingly, they are born directly, without a ganglion mother cell (GMC) intermediate, from the neuroblast, with the birth order of: Ap1/Nplp1, Ap2, Ap3, and Ap4/FMRFa (Figure 1H). Ap neurons are generated during the last part of a large (10 cell) Cas temporal window, where the four last-born cells also express Grh. *cas* plays a critical role during Ap neuron determination, and with the exception of Eya, expression of all Ap cluster determinants and terminal genes is lost in *cas* mutants. In spite of this seemingly broad function of *cas*, it triggers three regulatory events that, in turn, lead to the subdivision of the Ap window into three distinct windows. *cas* does so by activating *col*, and thus automatically the *col*→*ap/eya*→*dimm*→*Nplp1* feed-forward loop, as well as by simultaneously acting in a *cas*→*sqz*→*nab* feed-forward loop. The latter loop acts to suppress *col*, but only after *col* is allowed to perform its early postmitotic role—activation of *ap* and *eya*. The late down-regulation of *col* in later-born Ap cluster cells prevents the feed-forward action of *col*—specifying the Ap1/Nplp1 cell fate—and instead allows for the specification of the later-born Ap neuron cell fates. *sqz* and *nab* do not regulate the *cas* or *grh* temporal genes, but rather act downstream of *cas* to subdivide the Ap window. We propose that these genes be referred to as subtemporal genes by the definition that they act downstream of the canonical temporal genes, do not regulate temporal genes, and act to subdivide larger temporal windows. As anticipated from their temporal roles, *cas* also activates *grh* at the end of the Ap window, and *grh* represses *cas* in a negative feedback manner. However, *grh* also acts in an instructive manner and at high expression levels determines the Ap4/FMRFa cell fate.

In summary, the latter part of the NB 5-6T lineage ends with a large Cas window that is sequentially and combinatorially divided into subwindows, both by temporal gene expression levels and by a feed-forward-mediated timing device, consisting of two opposing feed-forward loops. Each subwindow triggers the expression of a unique set of postmitotic cell fate determinants that, in turn, dictates a unique neuronal cell fate. We speculate that the mechanism whereby a common upstream temporal cue triggers multiple opposing feed-forward loops is likely to be used by many stem cell lineages to generate cellular diversity.

RESULTS

The Lineage of Thoracic Neuroblast 5-6

In the embryonic *Drosophila* ventral nerve cord (VNC), each hemisegment contains ~30 neuroblasts in seven rows, identifiable by position, size, and molecular markers (Doe and Technau, 1993). Each individual neuroblast divides asymmetrically in a stem cell manner, in this way self-renewing while also producing smaller GMCs. Each GMC in turn divides once to generate two neurons or glia (Figure 1H) (Schmid et al., 1999; Schmidt et al., 1997).

To address the question of how one identified progenitor cell can generate different cell types, we resolved the complete lineage of the thoracic neuroblast 5-6 (NB 5-6T) and determined how the different Ap neurons emerge within this lineage. To this end, we utilized the NB 5-6–specific transgenic marker, *lbe(K)-lacZ* (De Graeve et al., 2004) and *lbe(K)-Gal4* (this study) (Figures 1F and 1G), combined with a number of other transgenic and antibody markers. These included markers for the temporal genes (Hb, Kr, Pdm, Cas, and Grh) (Brody and Odenwald, 2000; Isshiki et al., 2001; Kambadur et al., 1998) a marker for neuroblasts and early-born GMCs (Deadpan, Dpn) (Bier et al., 1992), markers for the previously identified Ap cluster determinants (Col, *sqz*, Nab, Dac, *ap*, Eya, Dimm, and phosphorylated Smad [pMad] as an ouput of BMP/TGF-β activation) (Allan et al., 2003, 2005; Baumgardt et al., 2007; Hewes et al., 2003; Miguel-Aliaga et al., 2004; Terriente Felix et al., 2007), as well as the terminal identity neuropeptide markers Nplp1 and FMRFa (Baumgardt et al., 2007; Benveniste et al., 1998; Park et al., 2004). In addition, markers for dividing cells (phospho-Ser10-HistoneH3; pH3) (Hendzel et al., 1997), for apoptotic cells (cleaved Caspase 3) (Nicholson et al., 1995), and BrdU labeling (Gratzner, 1982) were used to reveal common features of lineage progression. These lineage mapping results are described in detail in Figures S1–S4 available online.

To summarize the most pertinent points, the NB 5-6T neuroblast delaminates at late stage 8 and generates a lineage of 20 cells between embryonic stages 9 and 15. It then exits the cell cycle at stage 15 and dies via apoptosis at stage 16 (Figures 1H and S1–S3). The four Ap cluster neurons are born sequentially at the end of the lineage, with Ap1/Nplp1 first, the two "generic" Ap2 and Ap3 interneurons next, and Ap4/FMRFa last (Figure 1H). The neuroblast divides at regular intervals, and initially produces GMC daughter cells that each divides once to generate two postmitotic cells. However, at stage 13, as Ap1/Nplp1 is generated, there is a surprising switch in the division mode, and all four Ap neurons are generated directly from the neuroblast, without a GMC intermediate (Figures S3 and S4): This switch in division mode is similar to the end of the NB 7-3 lineage, where the last neuron is also generated without a GMC intermediate (Karcavich and Doe, 2005). NB 5-6T displays a canonical progression of temporal gene expression, except for a first phase of Pdm expression that persists in the two first-born cells. The lineage ends with a large, 10-cell, Cas window, where the four last-born cells also express Grh. These four last-born cells constitute the Ap cluster neurons (Figure 1H).

castor Plays Critical Roles during Apterous Neuron Determination

Why are the four Ap neurons specified at the end of the NB 5-6T lineage? To address whether the *cas* temporal gene is involved in this decision, we analyzed expression of all identified Ap neuron determinants in *cas* mutants. We found a complete loss of expression of the majority of determinants, including *ap*, Col, Dac, and Dimm in *cas* mutants (Figures 2E–2L). As anticipated from these effects, we found a complete loss of expression of the neuropeptides Nplp1 and FMRFa (Figures 2A–2D). Previous studies demonstrated a loss of *nab* expression in *cas* mutants (Clements et al., 2003), and similarly we found that Nab expression, as well as *sqz* expression, is lost in *cas* mutants (Figures 2M, 2N, and S5). Surprisingly, Eya expression is not lost, but rather deregulated in the VNC (Figures 2G–2J). In line with the maintained Eya expression, we found that the NB 5-6T lineage progresses normally, but displays an increase in the number of cells generated (Figure 2O). This is coupled with extended labeling for pH3 in the neuroblast into stage 16, a stage when we normally never see signs of mitotic activity (data not shown). Together, these results indicate that in *cas* mutants, the neuroblast fails to exit the cell cycle at stage 15. In addition, as anticipated from the regulatory interplay between *cas* and *pdm* (Kambadur et al., 1998) (*pdm* here refers to the two adjacent *nubbin/pdm1* and *pdm2* genes), we found that Pdm (Nubbin) expression is maintained for a prolonged period in *cas* (data not shown). Taken together, these results support a role for *cas* in suppressing *pdm*, in activating Ap neuron determinants, and in terminating the lineage progression of NB 5-6T.

In the Early Apterous Cluster Window, the Primary Role of *castor* Is to Activate *collier*

The temporal genes play critical roles in determining distinct windows of competence in neuroblasts, as evident by the loss or increase of certain cell types in temporal mutants (reviewed in Jacob et al., 2008). But what are the targets of the temporal genes? Previously, it has been speculated that temporal genes, such as *hb*, may regulate temporal transitions in neuroblast competence by modulating chromatin structure (Grosskortenhaus et al., 2005). The detailed mapping of the NB 5-6T lineage and the identified genetic cascades involved in determining cell identity of the different Ap neurons provide an opportunity to address this issue.

col is a critical determinant of early Ap neuron identity, and, as shown above, the expression of Col is completely lost in *cas* mutants. Together, this allowed us to address whether *col* mediates all the *cas* functions in the Ap cluster. Strikingly, when we reexpressed Col in *cas* mutants, we found robust reappearance of cells expressing *ap*, Dimm and Nplp1 (Figures 3A–3D). Previous studies revealed that Col is sufficient to trigger ectopic Ap neurons when misexpressed earlier in the NB 5-6T lineage, within the early Cas window, and as anticipated, *col* cross-rescue of *cas* results in supernumerary Ap1/Nplp1 and Ap2/3 cells (Figures 3A–3D). On the other hand, because *col* does not control Ap4/FMRFa-specific determinants, such as *sqz*, Dac, and Nab, that are also lost in *cas* mutants, we did not expect the *col* cross-rescue of *cas* to restore Ap4/FMRFa neurons. In line with this notion, in cross-rescued embryos we found no expression of FMRFa, *sqz*, Dac, or Nab in the NB 5-6T lineage (Figures 3D and S6).

These results demonstrate that *cas* acts through *col* to generate Ap1/Nplp1 and Ap2/3 neurons (Figure 3E). However, with respect to the generation of the Ap4/FMRFa neuron, *cas* plays additional roles, such as the activation of *sqz*, Dac, and Nab.

grainyhead Regulates *castor*, but Also Determines Ap4/FMRFa Cell Identity

Previous studies identified roles for *grh* in neuroblast cell cycle exit and apoptosis, but not in neuronal cell fate specification (Almeida and Bray, 2005; Cenci and Gould, 2005; Maurange et al., 2008). Consistent with these findings, in *grh* mutants we found an increase in the number of Ap neurons from four to six in the NB 5-6T lineage (Figures 4A–4F and 4K). In addition, however, we found clear effects upon Ap neuron specification, as evident by the loss of expression of Dimm, pMad, and FMRFa from the Ap4/FMRFa neuron (Figures 4A–4F and 4K). The loss of pMad could reflect a failure of the Ap4/FMRFa neuron to project its axon to its target gland, the dorsal neurohemal organ, with an accompanying failure to receive the TGF-β/BMP ligand Glass bottom boat. Indeed, by analyzing the axon of the Ap4/FMRFa neuron in *grh* mutants, we found a frequent loss of dorsal neurohemal organ innervation (75% failure to innervate in *grh* mutants, compared with 0% failure in control; $n > 52$ segments). These results argue for a combination of events, including a failure of the neuroblast to exit the cell cycle at stage 15, a failure to specify the last-born Ap neuron, the Ap4/FMRFa neuron, and an extension of the middle Ap neuron window, the Ap2/3 window (Figure 4J). In agreement with this notion, in *grh* mutants, we found no evidence for loss of expression of factors expressed by both Ap2/3 and Ap4/FMRFa, such as *sqz*, Nab, and Dac (Figures 4B and 4E; data not shown).

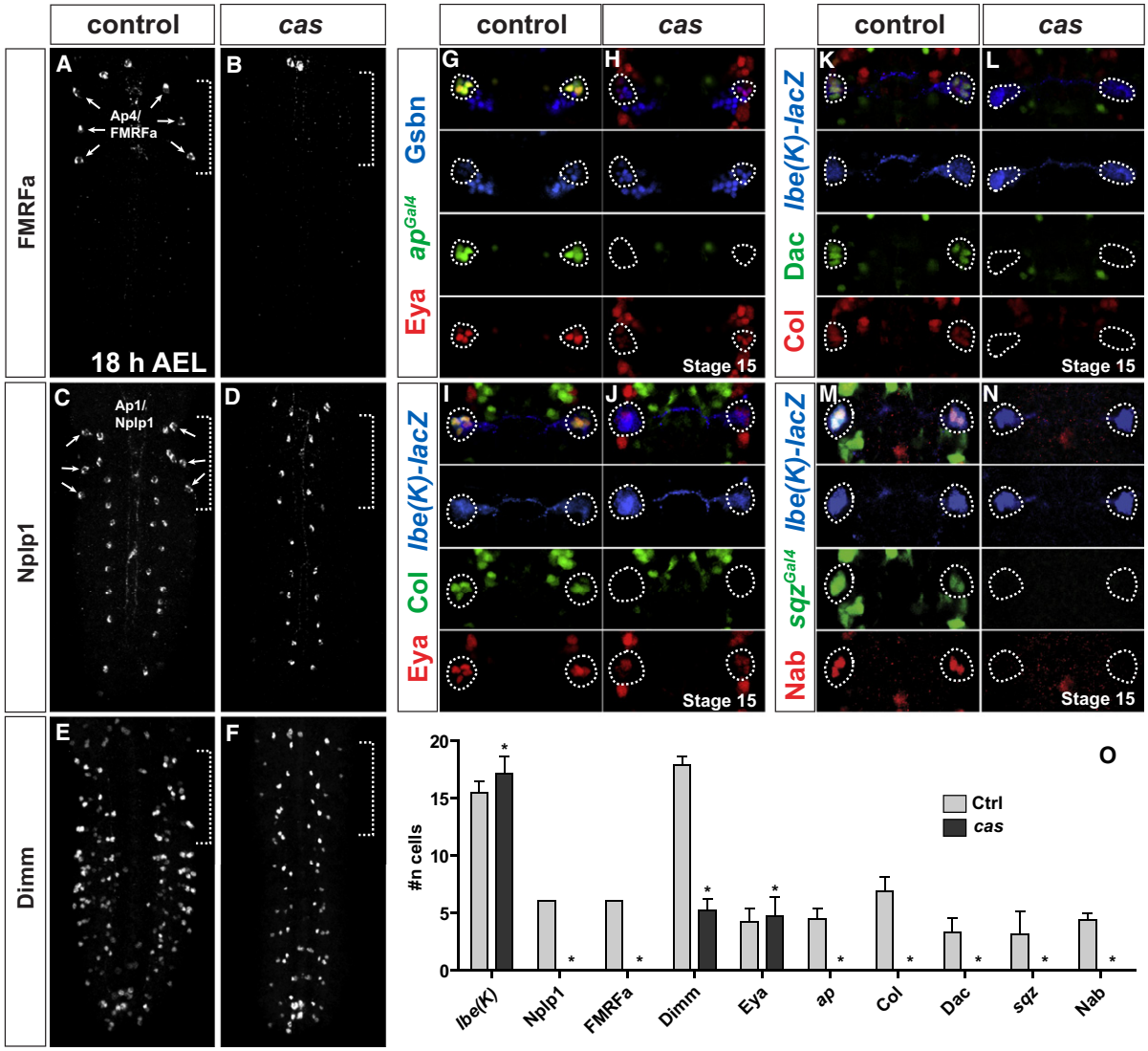


Figure 2. *castor* Plays Critical Roles during Ap Cluster Specification

(A–N) Expression of the terminal identity markers Nplp1 and FMRFa (A–D), and of the Ap cluster determinants Dimm, *ap*, Eya, Col, Dac, *sqz*, and Nab (E–N), in control and *cas* mutants.

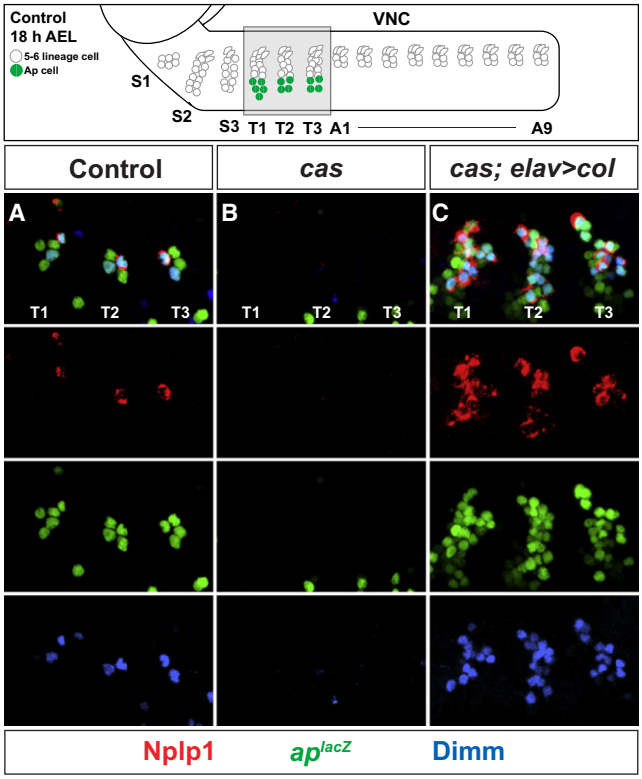
(A–F) Stage 18 hr AEL embryonic VNCs; anterior up; brackets outlining three thoracic segments. In control (A), FMRFa is specifically expressed in eight cells in the VNC: the six thoracic Ap4/FMRFa neurons, and the two SE2 neurons of the S2 segment. In *cas* (B), FMRFa is lost from the Ap4/FMRFa neurons, but not from the SE2 cells. In control (C), expression of Nplp1 is restricted to the 6 Ap1/Nplp1 neurons, and the 22 dorsal Ap neurons (dAps). In *cas* (D), expression of Nplp1 is specifically lost from the Ap4/Nplp1 neurons, whereas the dAps are unaffected. In control (E), the peptidergic determinant Dimm is expressed in peptidergic neurons in the VNC. In *cas* (F), the number of lateral Dimm expressing cells is reduced, both in the thorax and in the abdomen.

(G–N) Thoracic (T2) VNC segments, stage 15, with Gsbn (G and H) as a marker for the lineages of neuroblast rows 5 and 6, and *lbe(K)-lacZ* (I-N) as a marker for the NB 5-6T lineage. In control (G), the expression of *ap* and Eya defines the four Ap cluster cells, situated in the anterior- and lateral-most portion of the Gsbn compartment. In *cas* (H), expression of *ap* is lost from the Gsbn compartment; however, the expression of Eya is not lost but is ectopically expressed, both within the NB5-6T lineage, and globally within the VNC.

(I–N) Expression of Col (I–L), Eya (I and J), Dac (K and L), *sqz* (M and N), Nab (M and N), and *lbe(K)-lacZ* (I–N), within the NB5-6T lineage in control (I, K, and M) and *cas* (J, L, and N). In *cas*, expression of Col, Dac, *sqz*, and Nab, is lost from the NB 5-6T lineage. However, the number of *lbe(K)-lacZ* expressing cells is not reduced, indicating that NB 5-6T lineage cells are still generated.

(O) Quantification (n > 10 VNCs); data are represented as mean ± standard deviation (SD). Nplp1, FMRFa, and Dimm were quantified within lateral thoracic compartments. *ap* and Eya were quantified within the T2/T3 Gsbn compartment (#n cells/hemisegment). Col, Dac, *sqz*, and Nab were quantified within single T2/T3 *lbe(K)-lacZ* lineages (#n cells/lineage). *lbe(K)* was quantified as total number of cells within single T2/T3 *lbe(K)-Gal4* lineages. Asterisks (*) denote significant difference compared to control ($p < 0.01$).

Genotypes: (A, C, E, and G) w^{1118}. (B, D, F, and H) $cas^{\Delta 1}/cas^{\Delta 3}$. (I and K) *lbe(K)-lacZ*/*lbe(K)-lacZ*. (J and L) *lbe(K)-lacZ*/ *lbe(K)-lacZ*; $cas^{\Delta 1}/cas^{\Delta 3}$. (M) *lbe(K)-lacZ*, *UAS-nmEGFP*/*lbe(K)-lacZ*; sqz^{Gal4}. (N) *lbe(K)-lacZ*, *UAS-nmEGFP*/*lbe(K)-lacZ*; $cas^{\Delta 1}/cas^{\Delta 3}$,$sqz^{Gal4}$. (O) Genotypes as above, except for *lbe(K)* that is *lbe(K)-Gal4*, *UAS-nmEGFP*/+ for control, and *lbe(K)-Gal4*/*UAS-nmEGFP*; $cas^{\Delta 1}/cas^{\Delta 3}$ for *cas* mutants.



D

	Ctrl	*cas*	*cas, elav>col*
ap	4.03 ± 0.18	0.00 ± 0.00*	18.50 ± 2.20†
Nplp1	1.00 ± 0.00	0.00 ± 0.00*	7.38 ± 1.36 †
Dimm	2.00 ± 0.00	0.00 ± 0.00*	12.95 ± 2.39†
FMRFa	0.97 ± 0.18	0.00 ± 0.00*	0.00 ± 0.00
Dac	3.89 ± 0.32	0.00 ± 0.00*‡	0.00 ± 0.00 ‡
Nab	3.95 ± 0.21	0.00 ± 0.00*‡	0.00 ± 0.00 ‡

* significant difference compared to control ($p<0.01$)
† significant difference compared to *cas* mutant ($p<0.01$)
‡ quantified at stage 15

E

Figure 3. In the Early Ap Window, the Primary Role of *castor* Is to Activate *collier*

(A–C) Expression of *ap*, Nplp1, and Dimm in control (A), *cas* (B), and *cas* with panneural misexpression of *col* (C); side views of thoracic segments, dorsal up, anterior to the left, stage 18 hr AEL.

(A) In control, the expression of *ap* defines the Ap cluster, with the Ap1/Nplp1 neuron expressing Nplp1 and Dimm. Dimm is additionally expressed within the Ap4/FMRFa neuron.

(B) In *cas*, the expression of *ap*, Nplp1, and Dimm is lost from the NB 5-6T lineage. Moreover, Col is lost from the NB 5-6T lineage in *cas* mutants (Figure 2J and 2O).

(C) By reexpressing *col* in a *cas* background, using the panneural driver *elav-Gal4*, expression of the *col* downstream targets *Nplp1*, *ap*, and *dimm* are restored. As demonstrated elsewhere (Baumgardt et al., 2007), in addition to rescuing Ap neurons, this *Gal4* driver leads to ectopic *col* expression in the early *cas* window, and this triggers ectopic Ap1/Nplp1 and Ap2/3 neurons.

(D) Quantification; data are represented as mean number of expressing cells per Ap cluster ± SD (n > 30 clusters). Quantifications performed at stage 18 hr AEL, except for Dac and Nab, which were quantified at stage 15 (‡). Asterisks (*) denote significant difference compared to control; daggers (†) denote significant difference compared to *cas*; $p < 0.01$.

(E) Model for the genetic pathway for Ap1/Nplp1 cell specification, based on these results, as well as results from Baumgardt et al. (2007). The primary role for *cas* is to activate *col*, while *col* plays several downstream roles, acting in a feed-forward loop.

Genotypes: (A) ap^{lacZ}/+; *elav-Gal4*/+. (B) ap^{lacZ}/+; $cas^{\Delta 4}$, *elav-Gal4*/$cas^{\Delta 1}$. (C) *UAS-col*/ap^{lacZ}; $cas^{\Delta 1}$/$cas^{\Delta 4}$, *elav-Gal4*. (D) Controls as in (A), *cas* as in (B), *cas*, *elav* > *col* as in (C).

In line with the specific loss of the Ap4/FMRFa cell fate in *grh* mutants, we noticed that Grh expression showed a gradual increase in the Ap window, with lowest levels in the first-born neuron (Ap1/Nplp1) and highest in the last-born neuron (Ap4/FMRFa; Figures S2G and S2H). To test whether the high postmitotic levels of Grh observed in Ap4/FMRFa are instructive, we overexpressed it postmitotically in all four Ap neurons, using the ap^{Gal4} driver. We found that high-level, postmitotic expression of Grh triggers ectopic FMRFa expression in many or all Ap neurons and, thus, can act to convert all four Ap neurons into an Ap4/FMRFa cell fate (Figures 4G–4I and 4K). Previously, we demonstrated that combinatorial panneuronal misexpression of other Ap4/FMRFa determinants results in widespread ectopic FMRFa expression (Baumgardt et al., 2007). To test whether *grh* is able to act in such combinatorial codes, we misexpressed it throughout the CNS, alone and in combination with *ap* and *dimm*. We found that, although single misexpression of each regulator has no or limited effect, double comisexpression and, in particular, triple comisexpression of *grh* with *ap* and *dimm* results in striking ectopic FMRFa expression throughout the CNS (Figures 4L, 4N, 4P, and 4R). As anticipated from the normal expression of Nplp1 in *grh* mutants, this comisexpression resulted in no or limited ectopic Nplp1 neuropeptide expression (Figures 4M, 4O, 4Q, and 4R).

We examined the regulatory interaction between *cas* and *grh*, both within this lineage and within the entire VNC, and found a complete loss of Grh expression in *cas* mutants, both in NB 5-6T and elsewhere in the VNC, with the exception of Grh midline expression, which is unaffected (Figure S7). In contrast, in *grh* mutants, Cas expression is maintained for a longer period in the VNC, and Cas is not down-regulated in NB 5-6T at stage 16 (Figure S7). As anticipated from the mutant analysis, we found that *cas*/*grh* double mutants display the same phenotype as *cas* single mutants (i.e., a complete loss of all Ap neuron determinants and markers other than Eya; data not shown).

The *cas* and *grh* genetic analyses demonstrate that the generation of the four Ap neurons occurs within a *cas*/*grh* window and that both of these temporal genes play critical roles to control Ap neuron specification. Both *cas* and *grh* appear to control cell cycle exit in the neuroblast. *cas* furthermore plays a general role and controls several key Ap neuron determinants, whereas *grh* plays a restricted role, preventing the extension of the Ap2/3 window, and acting at elevated levels to postmitotically specify the Ap4/FMRFa fate. *grh* can even act in a combinatorial manner with other Ap4/FMRFa determinants to ectopically trigger FMRFa expression. *cas* and *grh* furthermore control each others expression by positive control (*cas* → *grh*) and negative feedback (*cas*⊣*grh*).

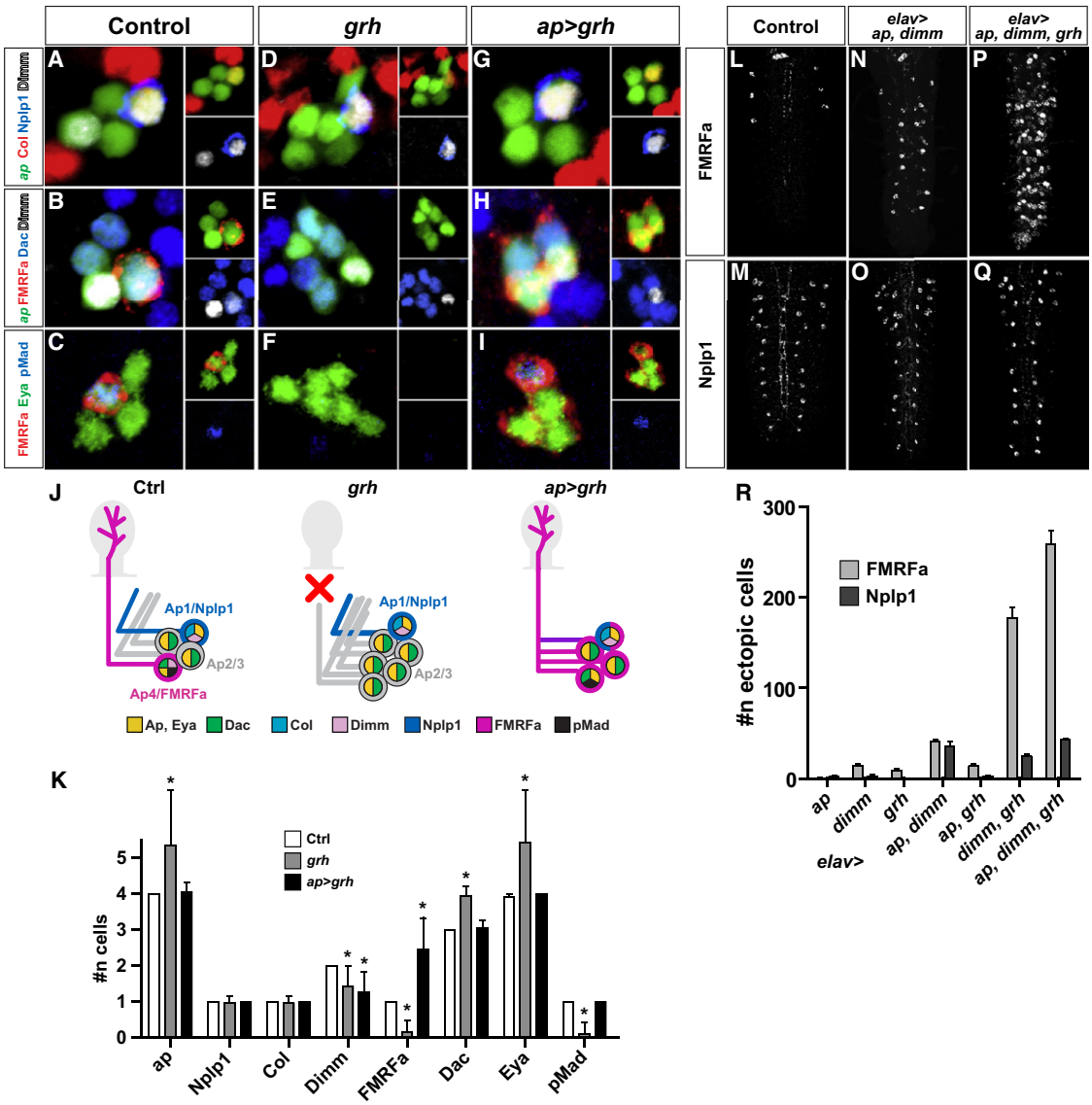


Figure 4. *grainyhead* Plays Multiple Roles during Ap Cluster Specification

(A–I) Expression of *ap*, Col, Nplp1, Dimm, FMRFa, Dac, Eya, and pMad in control (A–C), *grh* (D–F), and embryos overexpressing *grh* from the ap^{Gal4} driver (G–I). (A–I) Lateral views of Ap clusters; T2 segments, stage 18 hr AEL embryos.

(A–C) In control, Nplp1, Dimm, and Col are coexpressed within the Ap1 neuron (A). Dimm is also expressed together with FMRFa and Dac (B), as well as with pMad (C), in the Ap4/FMRFa neuron. Dac is additionally expressed within the Ap2 and Ap3 neurons (B).

(D–F) In *grh*, the expression of FMRFa (E and F), Dimm (E) and pMad (F) is frequently lost from the Ap4/FMRFa neuron. Also, there are 1–2 additional cells within the cluster, as defined by the ectopic expression of *ap* (D and E), and Eya (F); these cells also express Dac (E). However, expression of *ap*, Col, Nplp1, and Dimm within the Ap1 neuron (D) is unaffected.

(G–I) *grh* overexpression within postmitotic Ap neurons triggers ectopic expression of FMRFa, in many or all Ap cluster neurons. However, the expression of pMad (I), Dac (H), as well as of Col, Nplp1, and Dimm within the Ap1/Nplp1 neuron (G) is unaffected. Surprisingly, the expression of Dimm is commonly lost from the Ap4/FMRFa neuron (G and H).

(J) Model of observed phenotypes.

(K) Quantification; data are represented as mean number of expressing cells per Ap cluster ± SD. Asterisks (*) denote significant difference compared to control ($p < 0.01$, $n > 30$ clusters).

(L–Q) Expression of FMRFa and Nplp1 in control (L and M) and misexpression embryos (N–Q).

(N and O). In *ap/dimm* comisexpression embryos, FMRFa (N) and Nplp1 (O) are ectopically expressed in a limited set of cells in the VNC.

(P and Q) Triple comisexpression of *ap/dimm/grh* increases the number of FMRFa expressing cells.

(R) Quantification; data are represented as mean number of expressing cells per VNC ± SD ($n > 3$ VNCs).

Genotypes: (A and B) ap^{Gal4}/+; *UAS-nmEGFP*/+. (D and E) grh^{IM}, ap^{Gal4}/grh^{Df}, *UAS-nmEGFP*. (C) w^{1118}. (G) grh^{IM}/grh^{Df}. (G and H) ap^{Gal4}, *UAS-nmEGFP/UAS-grh*. (I) ap^{Gal4}/*UAS-grh*. (K) Genotypes as above. (L and O) w^{1118}. (M and P) *UAS-ap*, *UAS-dimm*/+; *elav-Gal4*/+. (N and Q) *UAS-ap*, *UAS-dimm*/+, *UAS-grh/elav-Gal4*. (R) *elav-Gal4* crossed to the indicated *UAS-cDNA* transgenes.

The Apterous Window Is Further Subdivided by the Subtemporal Genes *squeeze* and *nab*

Although *grh* plays instructive roles during Ap window subdivision by specifying the Ap4/FMRFa cell fate, this clearly does not explain the full spectrum of regulatory events needed to specify the three distinct Ap neuron cell types. Previous studies revealed that *col* plays a critical early postmitotic role and determines a ''generic'' Ap neuron identity by activating *ap* and *eya* (Baumgardt et al., 2007). Subsequently, *col* is down-regulated, and this allows for the terminal specification of the three later-born neurons—the Ap2, Ap3, and Ap4/FMRFa neurons. However, neither loss- nor gain-of-function of *grh* affects the critical and precise postmitotic down-regulation of *col* (Figures 4A, 4D, and 4G), and thus other mechanisms must be at play.

Indications into how *col* becomes down-regulated came from detailed analysis of the expression and function of the *sqz* and *nab* genes, both of which have been found to affect Ap neuron specification. The phenotype of *sqz* is complex, with an addition of Ap1/Nplp1 cells, a partial loss of FMRFa in Ap4, and an increase in Ap cell numbers, restricted to the first thoracic segment (Allan et al., 2003, 2005; Baumgardt et al., 2007). These phenotypes are partly due to the fact that *sqz* normally acts to down-regulate *col* specifically in the late-born Ap neurons (Ap2, Ap3, and Ap4). However, the role of *sqz* in down-regulating *col* in only the late-born Ap neurons did not match its apparent expression in all four Ap neurons. *nab* mutants show similar phenotypes (Terriente Felix et al., 2007), but *nab* has not been analyzed for its possible involvement in Ap window subdivision. Utilizing our lineage-specific marker (*lbe(K)-Gal4*), we addressed the expression and function of *sqz* and *nab* in more detail. We found that *sqz* expression commences in the neuroblast at stage 13 and is maintained thereafter, leading to *sqz* expression in all four Ap neurons. Nab expression commences in the neuroblast at stage 14, and Nab is thus coexpressed with *sqz* only in the three later-born Ap neurons (Figures 5A and S8). Similar to *sqz*, in *nab* mutants, we found that expression of Col is not properly suppressed within the later-born Ap neurons, and Col, Dimm, and Nplp1 are ectopically expressed (Figures 5B, 5C, 5E, 5F, and 5K). Conversely, misexpression of *nab* completely suppresses Col expression in the Ap1/Nplp1 neuron, and as an effect thereof completely suppresses Nplp1 expression (Figures 5H, 5I, and 5K). Other Ap neuron markers are not affected in *nab* mutants or by *nab* misexpression, showing that the role of *nab* is exclusively to suppress Col (Figures 5C, 5D, 5F, 5G, 5J, and 5K). Sqz and Nab physically interact (Terriente Felix et al., 2007), and Nab is a well-conserved transcriptional corepressor (Clements et al., 2003; Mechta-Grigoriou et al., 2000; Russo et al., 1995; Terriente Felix et al., 2007). Together, these findings suggest that a Sqz/Nab complex, established postmitotically within the three later-born Ap neurons, could act to suppress Col and thereby prevent Col's feed-forward function in specifying the Ap1/Nplp1 fate. To address whether the function of *nab* is completely dependent on *sqz*, we misexpressed *nab* in a *sqz* mutant background. As anticipated, in the absence of *sqz*, *nab* is unable to suppress Col expression and the Ap1/Nplp1 cell fate (Figure S9).

A *cas*→*sqz*→*nab* Feed-Forward Loop Acts as a Critical Timing Device Ensuring Proper Apterous Window Subdivision

Both *sqz* and *nab* are controlled by *cas* (Figures 2M and 2N) (Clements et al., 2003). How then is the critical delay in Nab expression, when compared to *sqz* expression, accomplished? To address this question, we analyzed the regulatory relationship between *cas*, *sqz*, and *nab*. We found that Nab expression is affected in *sqz* mutants, whereas *sqz* and Cas expression is unaffected in *nab* (Figures 6A, 6B, and 6E; data not shown). Thus, *nab* is downstream of both *cas* and *sqz*. Does *cas* activate *nab* only via activation of *sqz*, or does it act in a feed-forward manner together with *sqz* to activate *nab*? To answer this question, we first tested whether *sqz* could rescue *cas*, but found no evidence of cross-rescue (Figures 6C–6E). Next, we tested whether *cas* can ectopically activate *sqz* and Nab, whether *sqz* can ectopically activate Nab, and whether *cas*/*sqz* can combinatorially activate Nab. We found that *cas* can activate *sqz* (Figure S5) and that both *cas* and *sqz* can ectopically activate Nab (Figures 6F–6H and 6J–6L). However, we also found evidence of combinatorial activity, because *cas*/*sqz* comisexpression results in increased ectopic Nab expression, compared with *cas* or *sqz* single misexpression (Figures 6I, 6M, and 6N).

DISCUSSION

This study has focused on one identified *Drosophila* neural progenitor cell and its lineage, the thoracic neuroblast 5-6, with particular emphasis on the temporal transitions acting to dictate several unique cell fates at the end of this lineage. We find a remarkable complexity in regulatory interactions, where combinatorial events and feed-forward loops act in sequence to govern high-fidelity cell fate specification of the different Ap neurons. At the top of this hierarchy is the temporal gene *cas*, which acts as a key trigger of the Ap window, by simultaneously activating *col*, *sqz*, and *grh* (Figure 7A). This triple gene activation sets in motion a cascade of regulatory events: (1) a *col*→*ap*/*eya*→*dimm*→*Nplp1* feed-forward loop, (2) an opposing *cas*→*sqz*→*nab* feed-forward loop, and (3) a gradual increase in Grh levels, culminating in the last-born cell. In addition, *cas* also activates *dac* in all four Ap cluster neurons. The precise regulatory dynamics of these events and the NB 5-6T lineage progression act in concert to subdivide the larger Cas window, and to ensure that precisely four Ap neurons are generated with three distinct cellular identities.

Temporal and Subtemporal Regulators

There are several key features that signify the canonical temporal gene cascade (*hb-Kr-pdm-cas-grh*) (Brody and Odenwald, 2002; Jacob et al., 2008). First, they are expressed by and act in most, if not all, neuroblasts. Second, they regulate each other. Third, they act to specify a multitude of cell types, including glia, interneurons, and motoneurons. Subtemporal genes differ from temporal genes in all of these aspects: they act downstream of temporal genes, they do not regulate the temporal genes, they act to subdivide larger temporal windows, and they may be expressed by and act only in subsets of

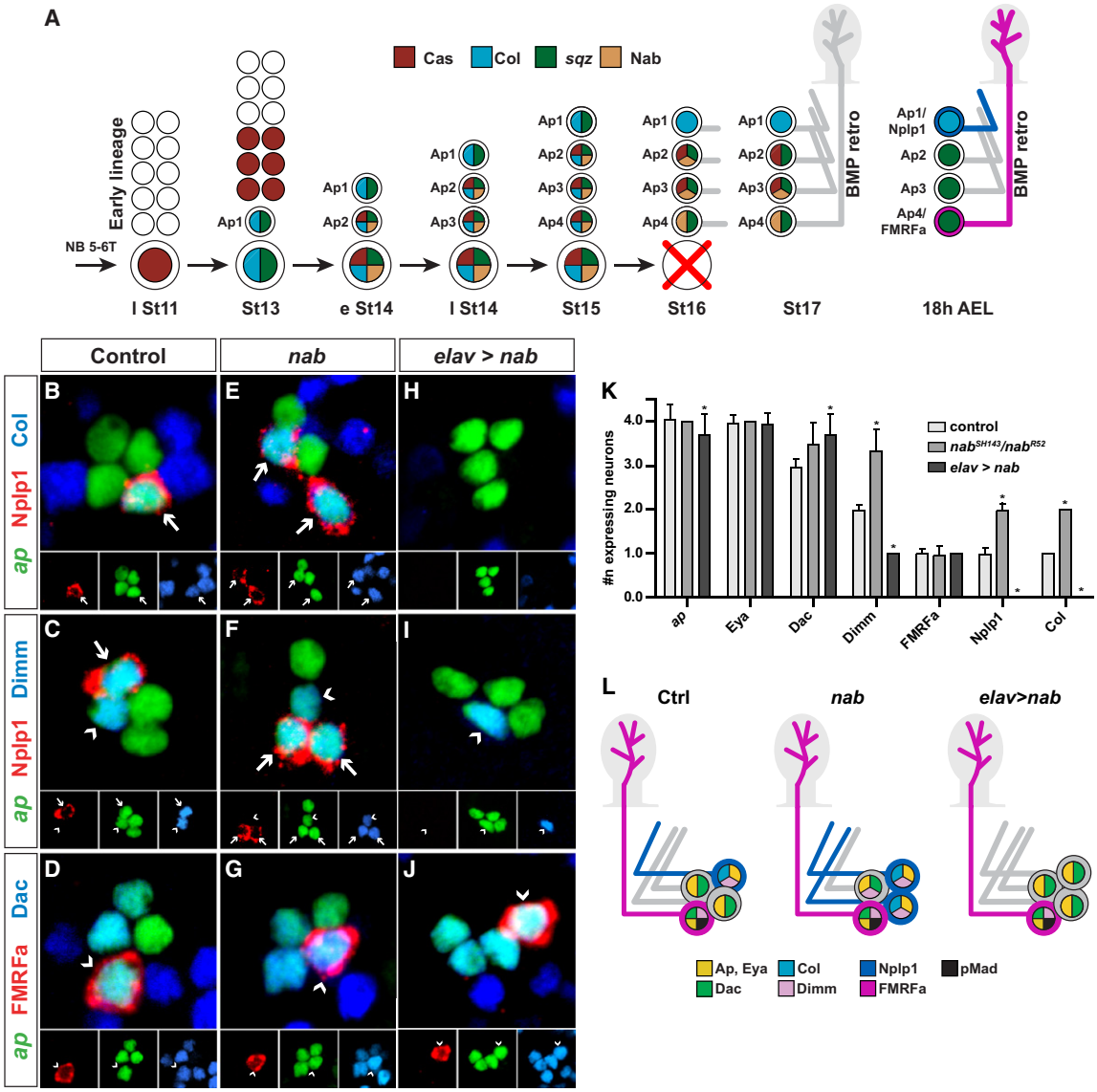


Figure 5. *nab* Plays a Critical Role in Suppressing *collier*

(A) Expression of Cas commences within NB 5-6T at stage late 11, and an early Cas window consisting of the progeny of three GMCs is generated. At stage 13, levels of Cas is reduced beyond detection, and the neuroblast begins expressing Col and *sqz*. This leads to the generation of a neuron that expresses both Col and *sqz*, but neither Cas nor Nab—the prospective Ap1/Nplp1 neuron. The generation of this neuron coincides with a switch in the mode of division of the neuroblast to generating neurons directly, without a GMC intermediate. At early stage 14, the expression of Nab commences in the neuroblast. Nab is subsequently coexpressed with Cas, Col, and *sqz* during the generation of the three late Ap cluster neurons: Ap2, Ap3, and Ap4/FMRFa. After stage 15, the expression of Cas and Nab is gradually lost from the postmitotic neurons, whereas Col is specifically down-regulated from all but the Ap1/Nplp1 neuron.

(B–J) Expression of *ap*, Nplp1, Col, Dimm, FMRFa, and Dac, in control (B–D), *nab* (E–G), and *nab* misexpression embryos (H–J); T2 clusters, stage 18 hr AEL embryos.

(B–D) In control, Nplp1, Col, and Dimm are specifically coexpressed within the Ap1/Nplp1 neuron (B and C, arrow). Dimm is additionally coexpressed with FMRFa and Dac within the Ap4/FMRFa neuron (C and D, arrowheads). Dac is also expressed within the Ap2 and Ap3 neurons, but not in the Ap1/Nplp1 neuron (D, arrow).

(E–G) In *nab*, one additional Col/Nplp1 expressing cell is evident within the Ap cluster (E and F, arrows) and Dimm is now expressed in 3–4 cluster neurons (F). Expression of FMRFa and Dac, on the other hand, is unaffected (G).

(H–J) When *nab* is misexpressed, using the late panneural driver *elav-Gal4*, expression of Nplp1 and Col is lost from the Ap cluster (H and I). Additionally, expression of Dimm is lost from one of the Ap cluster neurons (I). Dac on the other hand is frequently ectopically activated within all four Ap cluster neurons, whereas the expression of FMRFa remains unaffected (J).

(K) Quantification; data are represented as mean number of expressing cells per Ap cluster ± SD (n > 30 clusters). Asterisks (*) denote significant difference compared to control ($p < 0.01$).

(L) Model of the observed phenotypes.

Genotypes: (B–D) ap^{lacZ}/+. (E–G) ap^{lacZ}/+. nab^{SH143}/nab^{R52}. (H–J) *UAS-nab/+; elav-Gal4/+*. (K) Genotypes as above.

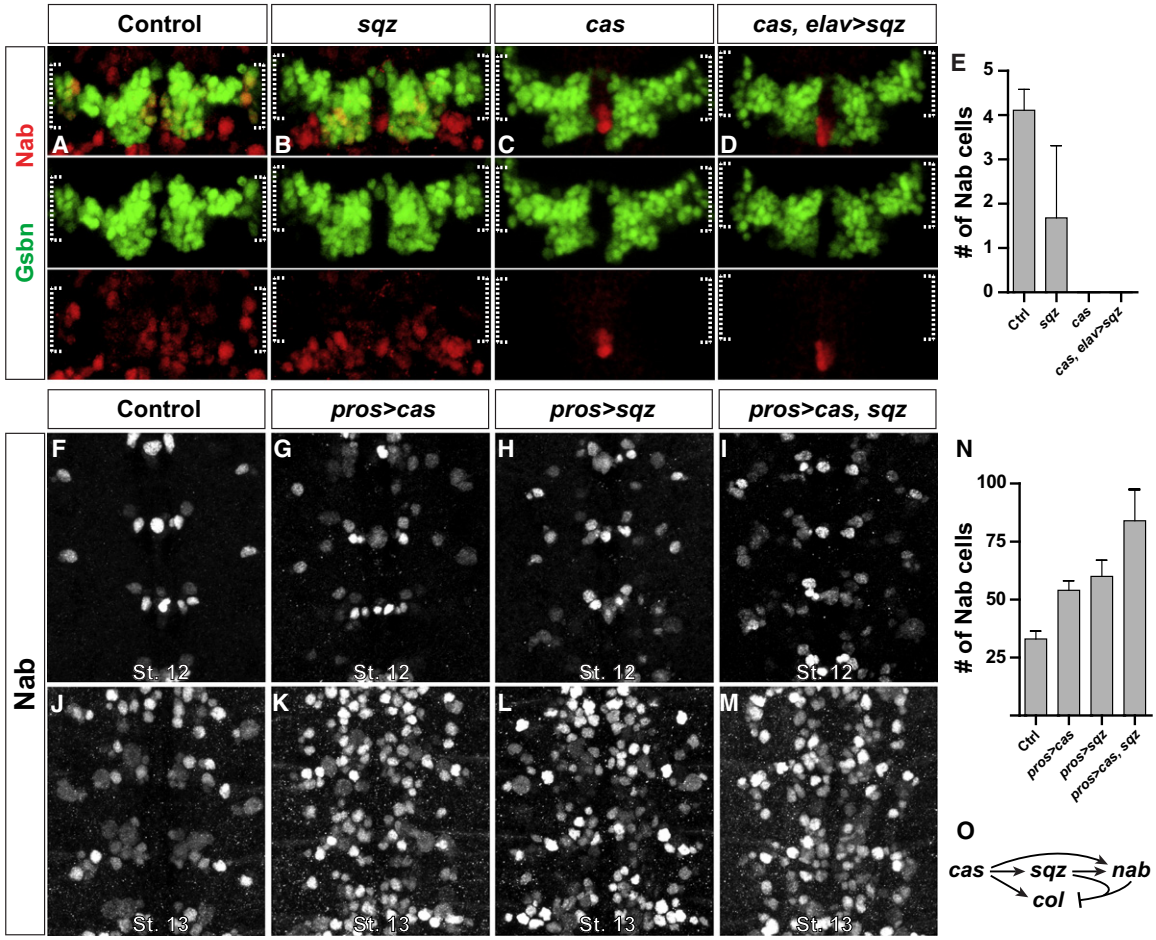


Figure 6. *cas* and *sqz* Act in a Feed-Forward Loop to Activate *nab*

(A–D) Expression of Nab in control, *sqz*, *cas*, and *sqz* "cross-rescue" embryos; T2 segments (anterior up), stage 15, with Gsbn as a marker for neuroblast rows 5 and 6.

(A) In control, Nab is expressed within late cells of the NB 5-6T lineage, situated lateral-most within the Gsbn compartment.

(B) In *sqz*, expression of Nab is frequently lost from the NB 5-6T lineage neurons.

(C) In *cas*, expression on Nab is lost from the embryonic VNC, except for a few cells at the VNC midline.

(D) Panneural expression of *sqz* in a *cas* mutant fails to restore Nab expression.

(E) Quantification; data are represented as mean number of Nab expressing cells within the lateral Gsbn compartment ± SD (n > 10 VNCs). Asterisks (*) denote significant difference compared to controls ($p < 0.01$).

(F–M) Expression of Nab in control (F and J), *cas* misexpression (G and K), *sqz* misexpression (H and L), and *cas/sqz* comisexpression VNCs (I and M), using the early neuroblast driver *pros-Gal4*. T2 and T3 segments; stage 12 (A, C, E, and G), and 13 (B, D, F, and H) embryos.

(F and J) In control, expression of Nab becomes evident in a few neuroblast lineages in the VNC at stage 12 (F), with additional lineages expressing Nab at stage 13 (J).

(G–H and K–L) Misexpression of either *cas* or *sqz* alone, leads to ectopic activation of Nab within a number of cells within the VNC.

(I and M) Comisexpression of *cas* and *sqz* leads to increased activation of Nab.

(N) Quantification; data are represented as mean number of Nab expressing cells within the T2 + T3 segments of stage 12 embryos ± SD (n > 4 embryos).

(O) Model showing the regulatory relationship between *cas*, *sqz*, *nab*, and *col*. Initially, *cas* activates both *sqz* and *col*. However, after *cas* and *sqz* together have activated *nab*, *col* is down-regulated from the late-born, postmitotic Ap neurons by the concerted action of *sqz* and *nab*. This delay allows for *col* to play its important transient role—activation of *ap* and *eya*.

Genotypes: (A) w^{1118}. (B) sqz^{IE}/sqz^{Df2411}. (C) $cas^{\Delta 1}/cas^{\Delta 4}$. (D) ap^{lacZ}/UAS-*sqz*; $cas^{\Delta 1}/cas^{\Delta 4}$, *elav-Gal4*. (E) Genotypes as in (A–D). (F and J) w^{1118}. (G and K) *pros-Gal4/UAS-cas*. (H and L) *pros-Gal4/UAS-sqz*. (I and M) *pros-Gal4/UAS-sqz*; *UAS-cas*/+. (N) Genotypes as in (F)–(M).

neuroblasts. In addition, as an effect of their more restricted expression, they may be responsible for dictating only certain types of cell fates. For instance, we have found no evidence of *sqz* expression in glia or motoneurons (Allan et al., 2003), indicating that this gene may primarily act as a subtemporal gene during interneuron specification. Interestingly, the stepwise refinement of temporal windows described here (i.e., temporal genes acting on subtemporal genes) is reminiscent of the manner in which early embryonic patterning cues are gradually refined by the subsequent activation of increasingly restricted positional cues (Jessell, 2000; Skeath and Thor, 2003).

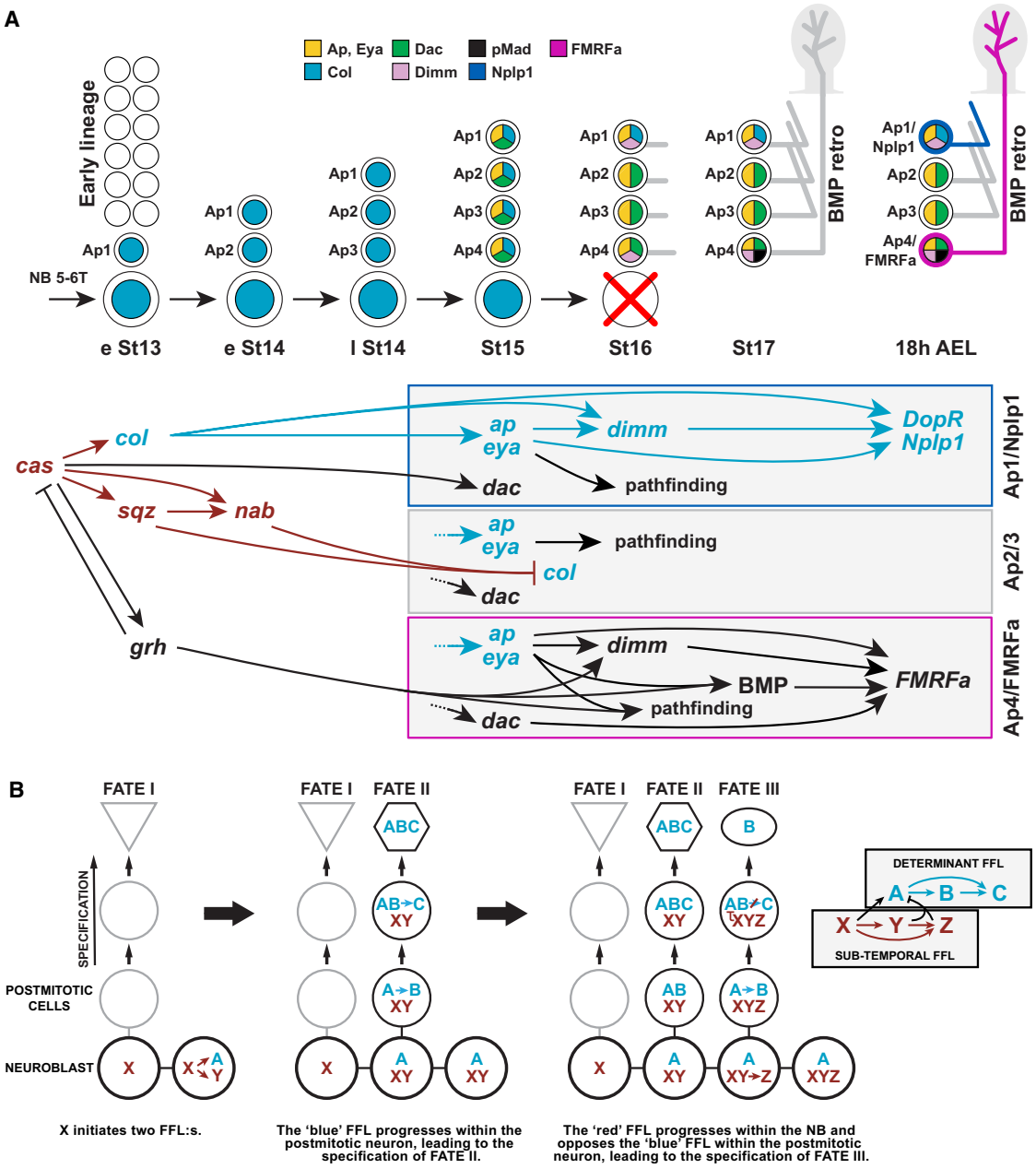


Figure 7. Specification of Ap Cluster Neurons in NB 5-6T

(A) Cartoon outlining lineage progression and gene expression (top), as well as the regulatory cascades (bottom) acting to specify the distinct Ap neuron cell fates in the NB 5-6T lineage (based on this and previous studies; see text for references). The temporal gene *cas* triggers four regulatory events: the *col*→*ap/eya*→*dimm*→*Nplp1* feed-forward loop (blue), the *cas*→*sqz*→*nab* feed-forward loop (red), and the expression of the temporal gene *grh* and the determinant *dac*. The regulatory interplay between these events allows for *col* to play its early role—specifying a "generic" Ap neuron fate in all four Ap neurons—but prevents the *col*→*ap/eya*→*dimm*→*Nplp1* feed-forward loop progressing in the three later-born Ap neurons. Dac and increasing levels of Grh acts to ensure the final fate of the last-born neuron, Ap4/FMRFa. See Results and Discussion for details.

(B) Model for how two opposing feed-forward loops, progressing within a lineage, can control the generation of distinct cell fates at each division. Terminal cell FATE I is specified by previous regulatory events (left). The upstream regulator *X* simultaneously activates two different feed-forward loops (FFL): *X*→*A*→*B*→*C* (blue) and *X*→*Y*→*Z* (red). The blue loop progresses via a transient AB cell fate into the final ABC fate (FATE II) (middle). The progression into FATE II within the last-born cell is blocked by the progression of the red feed-forward loop, but only after the transient fate (AB) was established (right). This allows for an alternative cell fate (FATE III) to be established in the last-born cell. (boxes, right) The two feed-forward loops are different in their nature; the blue "late determinant" loop actively specifies a unique terminal cell fate, while the red "subtemporal" loop opposes the determinant loop.

The Downstream Targets of Temporal and Subtemporal Genes

Given that the temporal genes appear to act in most, if not all, neuroblast lineages to specify a wide range of cell types, it has been postulated that they may play these diverse roles by altering chromatin states (Grosskortenhaus et al., 2005). In such a model, Cas, for instance, would act to ensure that "late genes," whether general or subtype-specific genes, would be kept in an open and accessible chromatin state, but Cas would never directly regulate any late gene. Although we subscribe to this general notion, our finding that Ap neuron cell fates can be restored in *cas* mutants simply by reexpressing *col* suggests that temporal genes may also act in a more direct regulatory manner to control cell fate determinants. Specifically, if *cas* was critical for establishing a "late chromatin landscape," it is unlikely that reexpression of *col* alone could trigger activation of the Ap neuron determinants. Rather, our data suggest direct regulation of *cas* upon the cell fate determinant *col*. In addition, because *col* is not lost in the entire VNC, the activation of *col* by *cas* appears to be lineage specific, and thus context dependent. Moreover, our findings that the last temporal gene in the canonical cascade, *grh*, is present at high levels in postmitotic Ap4/FMRFa neurons and acts to activate FMRFa expression both in Ap neurons and ectopically in many CNS neurons, suggest that, in certain contexts, temporal genes may even play postmitotic roles during cell fate specification and act directly upon terminal identity genes. Perhaps the potency of temporal genes to control diversity in a wide spectrum of neuroblast lineages results from a multifaceted range of functions, including controlling chromatin state, directly regulating cell fate determinants, and even directly regulating terminal identity genes. Alternatively, because no postmitotic role has been ascribed for the early temporal genes *hb* and *Kr*, it is possible that different temporal genes control lineage diversity in different manners.

Opposing Feed-Forward Loops Provide High-Fidelity Control of Neuronal Specification and Cell Numbers

Studies of gene regulatory networks in *Escherichia coli* and yeast have revealed a common use of the so-called coherent feed-forward loop, whereby gene *X* activates gene *Y* and then acts with gene *Y* to activate gene *Z*, resulting in an $X \rightarrow Y \rightarrow Z$ loop (Milo et al., 2002; Shen-Orr et al., 2002). Recently, such feed-forward loops have been identified in the developing nervous system (Baumgardt et al., 2007; Johnston et al., 2006), and they are likely to be a common feature of many genetic networks acting to specify neuronal subtype identities. The studies presented here reveal a novel genetic mechanism, involving an elaboration of the coherent feed-forward loop, whereby a common upstream regulator, *cas*, simultaneously triggers not one but two distinct feed-forward loops. One loop is allowed to progress to control a generic and transient cell fate in all cells, only to later be blocked in subsets of cells by the progression of the second loop (Figure 7B). The loops are different in nature; the "blue" loop (*col*→*ap/eya*→*dimm*→*Nplp1*, or $A \rightarrow B \rightarrow C$ in Figure 7B), involves instructive cell fate determinants progressing toward a terminal cell fate (Ap1/Nplp1 or FATE II in Figure 7B). In contrast, the "red" loop involves subtemporal regulators that act to block the "blue" loop. It is tempting to speculate that this novel mechanism of simultaneously triggered opposing feed-forward loops will be identified in many other neural lineages. Although complex in their nature, simultaneously triggered opposing feed-forward loops can perhaps be viewed as a logical extension of the basic coherent feed-forward loop identified in single cell organisms, an extension necessitated by the evolution of complex and large nervous systems in metazoans.

However, for this mechanism to work efficiently, parts of each feed-forward loop must be restricted to progenitor or postmitotic cells, respectively. Specifically, activation of *nab* (or *Z* in Figure 7B) in the *cas*→*sqz*→*nab* loop must occur only in the neuroblast; otherwise, *nab* would eventually be up-regulated also in the first-born Ap neuron, the Ap1/Nplp1, and suppress the *col* feed-forward loop in this neuron. Conversely, the activation of the *col*→*ap/eya*→*dimm*→*Nplp1* feed-forward loop (or $A \rightarrow B \rightarrow C$ in Figure 7B) can occur only in postmitotic cells; otherwise, *col* would trigger the Ap1/Nplp1 terminal cell fate in the neuroblast. The mechanisms by which *nab* can only be activated in the neuroblast and the *col* feed-forward loop only act in postmitotic Ap neurons is unclear, but may result from the selective expression of other regulators, and/or from the global regulatory differences between stem cells and neurons currently being identified (Atkinson and Armstrong, 2008; Pietersen and van Lohuizen, 2008; Yoo and Crabtree, 2009).

EXPERIMENTAL PROCEDURES

Fly Stocks

UAS-grh transgenic flies were generated by inserting the *grh-O'* splice variant cDNA (Uv et al., 1997) (provided by A. Uv) into the pUASt vector (Brand and Perrimon, 1993). Misexpression of Grh from this construct was verified by crossing *UAS-grh* transgenes to *elav-Gal4* and staining using Grh antibodies. To generate the *lbe(K)-Gal4* transgenes, the enhancer fragment "K" from the ladybird early gene (De Graeve et al., 2004) (provided by K. Jagla) was inserted into the *P* element Gal4 plasmid, pMB3 (Certel and Thor, 2004). Transgenes were generated by standard procedures at BestGene. Other fly stocks are described in Supplemental Experimental Procedures.

Immunohistochemistry and In Situ Hybridization

In situ hybridizations was conducted as previously described (Tautz and Pfeile, 1989), using a cDNA covering the entire *sqz* coding region (Allan et al., 2003). Grh antibodies were raised against the C-terminal 135 amino acids. For c-Myc and proFMRF IgY antibodies, peptides for the c-Myc epitope (MEQKLI SEEDLNE) or the C-terminal part of proFMRF (GAQATTTQDGSVEQDQFFGQ) were injected into hens. For more details and for other antibodies used see Supplemental Experimental Procedures.

Confocal Imaging and Data Acquisition

Zeiss LSM 5 or Zeiss META 510 Confocal microscopes were used to collect data for all fluorescent images; confocal stacks were merged using LSM software or Adobe Photoshop. Where immunolabeling was compared for levels of expression, wild-type and mutant tissue was stained and analyzed on the same slide. Bright-field images were collected on a Nikon E400 microscope with a SPOT-RT digital camera. Statistical analysis was performed using Microsoft Excel, and bar graphs generated using GraphPad Prism software.

Statistical Methods

Quantifications of observed phenotypes were performed using Student's two-tailed t test, assuming equal variance.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and nine figures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)01358-0.

ACKNOWLEDGMENTS

We are grateful to S. Cohen, M. Crozatier, A. DiAntonio, C.Q. Doe, L. Fessler, A. Gould, R. Holmgren, K. Jagla, T. M. Laufer, W. Odenwald, R. Pflanz, J. Skeath, P.H. Taghert, A. Uv, D. Vasiliauskas, A. Vincent, the Drosophila Genomic Resource Center, the Developmental Studies Hybridoma Bank, and the Bloomington Stock Center for sharing antibodies, fly lines, and DNA. We thank D. van Meyel, I. Miguel-Aliaga, A. Gould and J.B. Thomas for critically reading the manuscript. H. Ekman and A. Angel provided excellent technical assistance. This work was supported by the Swedish Research Council, the Swedish Strategic Research Foundation, the Knut and Alice Wallenberg foundation, the Swedish "Hjärnfonden," "Cancerfonden," the Swedish Royal Academy of Sciences (support to S.T.), and the Spanish Ministerio de Ciencia e Innovación (grant BFU2008-04683-CO2-01/BMC to F.J.D.).

Received: June 29, 2009
Revised: August 27, 2009
Accepted: October 1, 2009
Published: November 25, 2009

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